Combination of Irreversible Electroporation with Checkpoint Blockade and Toll-like Receptor 7 Stimulation Induces Anti-Tumor Immunity in a Murine Pancreatic Cancer Model.

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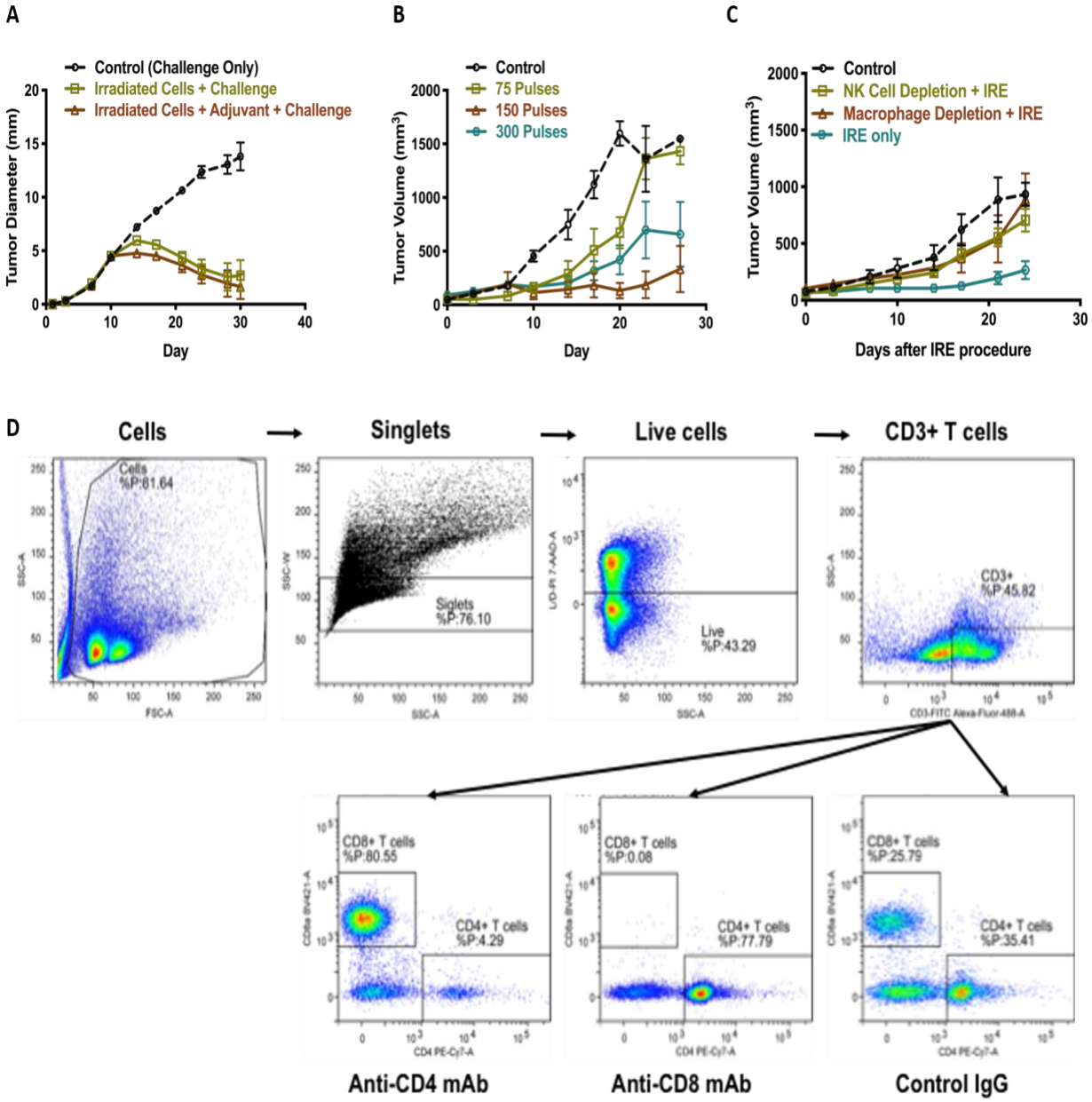
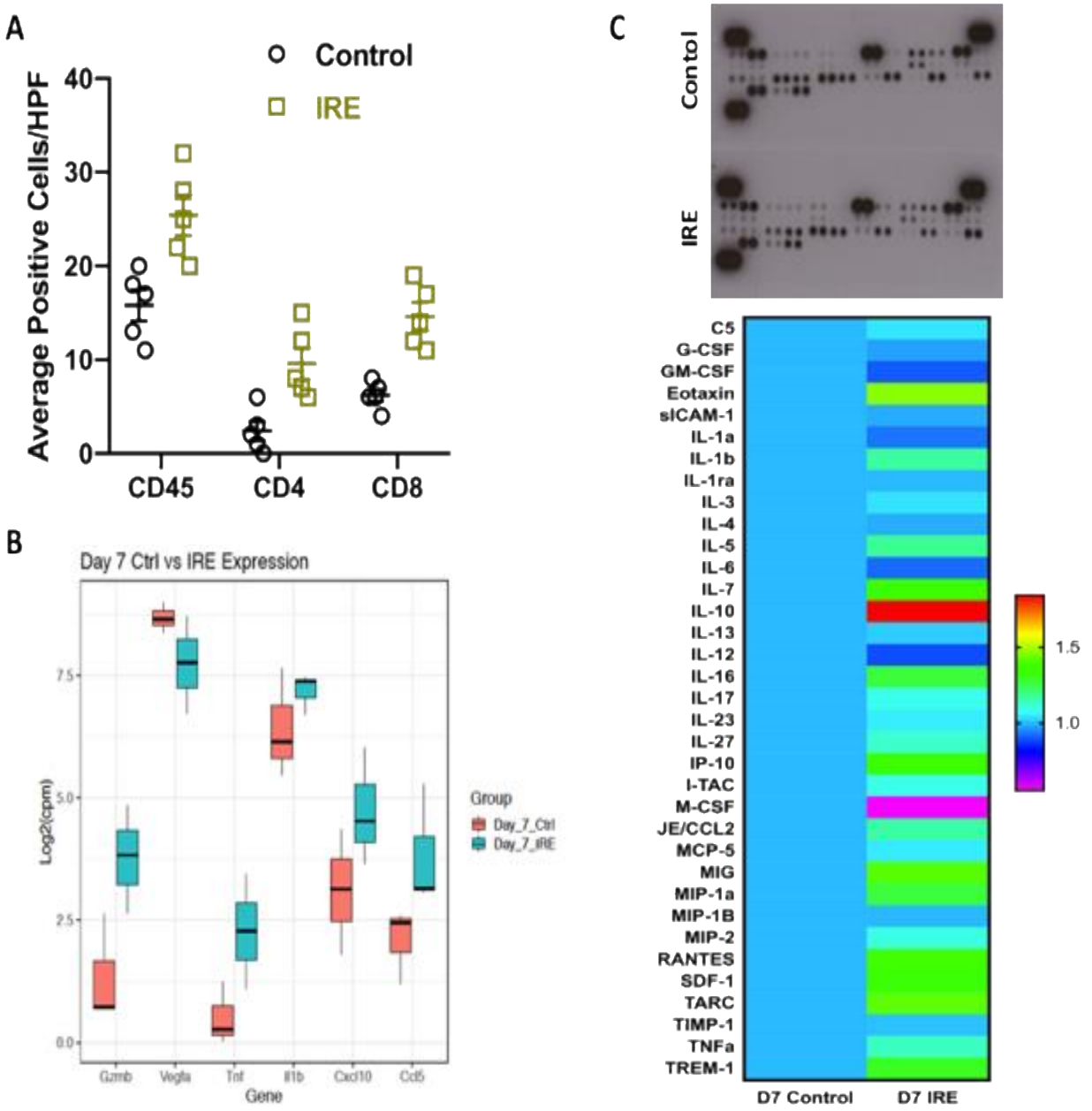
Supplementary Results

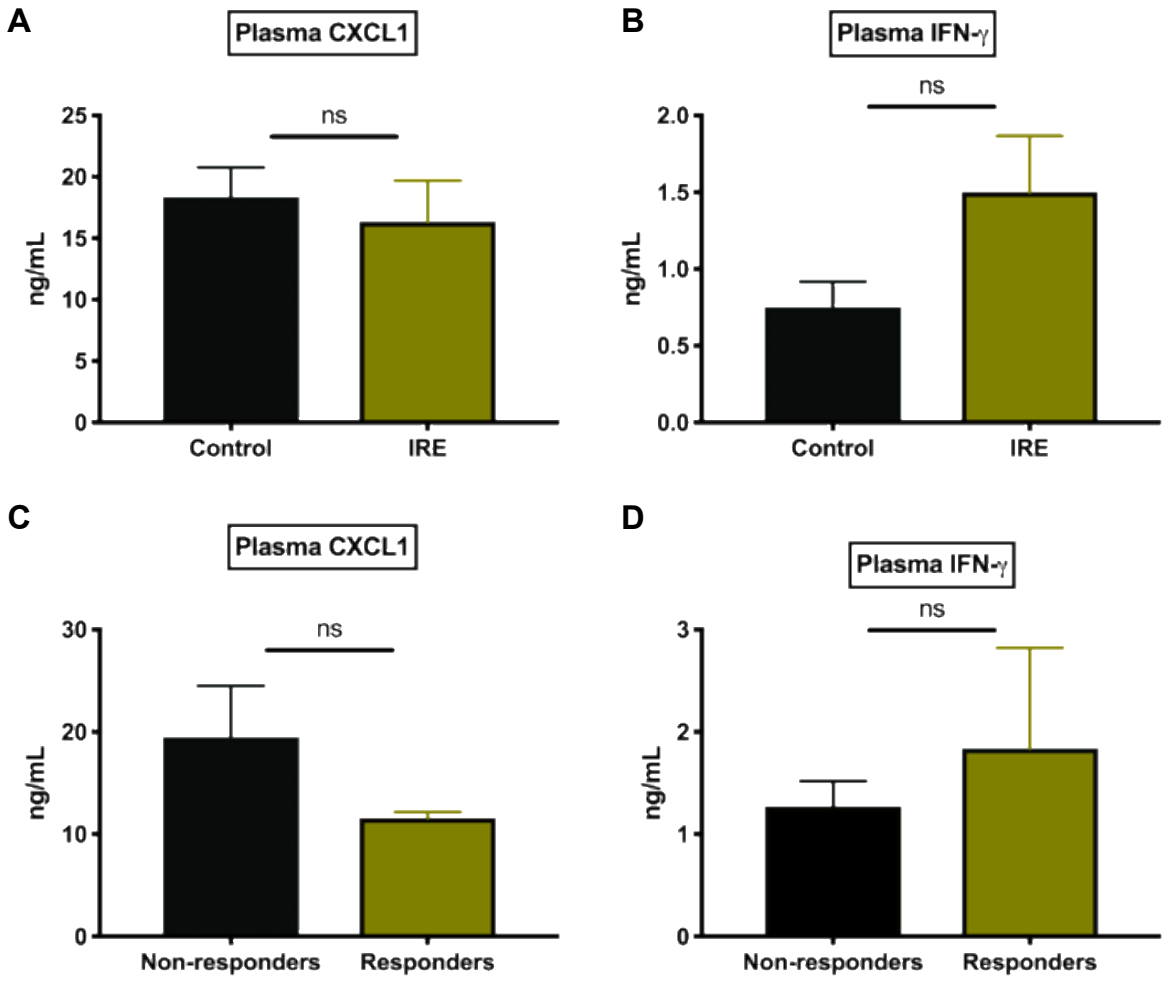


Supplementary Figure 1. (A) Growth curve of SQ KPC4580P tumors in control or vaccinated C57BL/6 mice showing the immunogenicity of the KPC4580P cell line. Immunocompetent C57BL/6 mice were immunized by injecting SQ either saline (Control) or 10^6 lethally irradiated KPC4580P cells (Vaccinated) with or without adjuvant (Poly I:C) for 14 days. The mice were challenged with live KPC4580P cells SQ on the contralateral flank and the tumor growth rate was monitored. **(B)** Tumor growth of SQ KPC tumors in immunocompetent C57BL/6 mice in response to different doses of IRE pulses treated when the tumor sizes were 5-6 mm diameter. **(C)** Tumor growth in mice depleted for NK cells or macrophages (the data for control and IRE groups are the same as from Figure 1F). NK cells were depleted by IP injection of 50 µg anti-mouse Asialo-GM1 antibody (Wako) every 5 days starting 6 days before tumor implantation. Macrophages were depleted by IP injection of 100 µL of chlodronate liposome (Liposoma) every 3 days starting 6 days before tumor implantation. **(D)** Flow cytometry data showing successful antibody mediated depletion of CD4 and CD8 T cells in mice on day 7 used in the immune cell depletion study represented in Figure 1F.

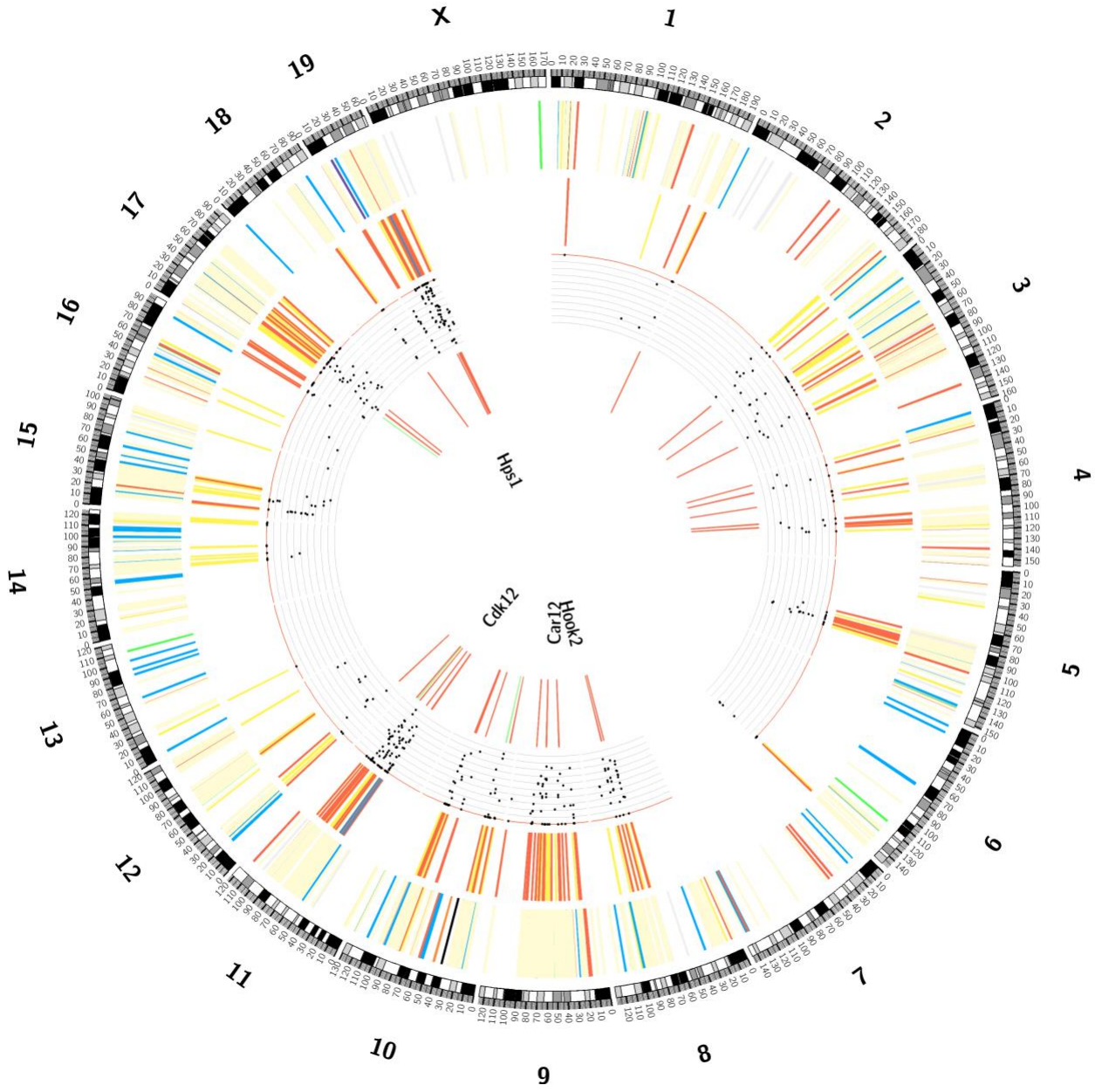


Supplementary Figure 2. (A) Quantitative interpretation of immune cell infiltration in tumor tissues using IHC. Tumors harvested from mice 7 days post treatment were fixed and IHC was performed to identify CD45, CD4 and CD8 positive immune cell information with representative images provided on Fig 2A. Each stained tissue section was analyzed under 40 x magnification and the number of positive cells under that particular high-power field (HPF) were counted. An average of the number of positive cells per field is represented as mean ± SEM. **(B)** RNA seq analysis of differentially expressed genes relevant to the immunological function post IRE treatment. Briefly, RNA-Seq data from 3 replicates for each group were aligned to the mm10 mouse genome using the STAR aligner and assigned to transcripts and genes using RSEM. Count data were input into an edgeR DGE object, normalized with TMM, filtered for non-expressed genes, and

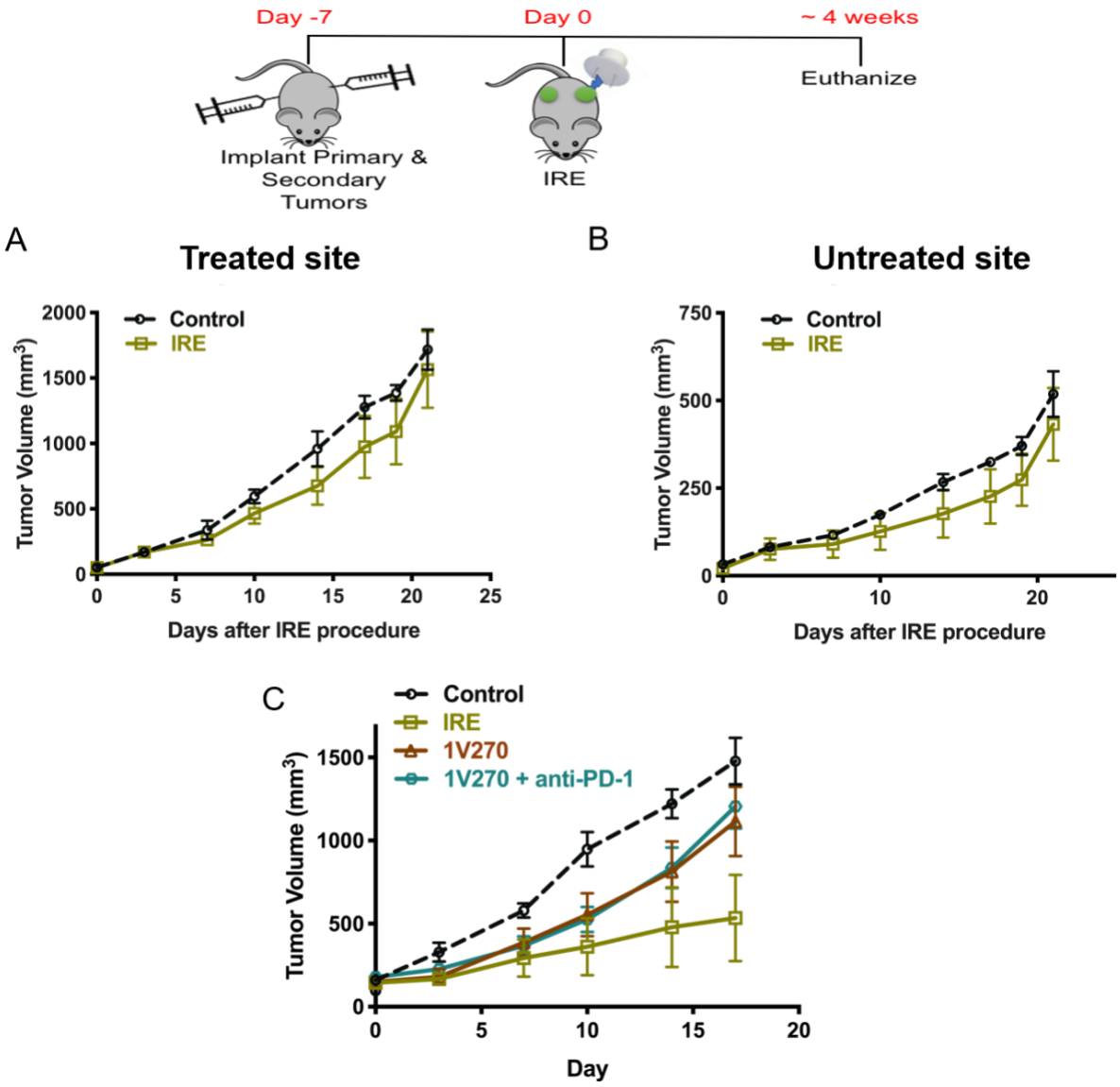
analyzed for differential gene expression between the two groups with limma (Ref 5). *Gzmb*, *Vegfa*, and *Tnf* had unadjusted limma p-values less than 0.05, and *Il1b*, *Cxcl10*, and *Ccl5* did not. **(C)** Image of the array blots (Top) and heatmap of relative fold change in the cytokine expression levels (Bottom) normalized to the control samples at the same timepoint (7 days post treatment) analyzed using Multiplex Proteome Profiler Cytokine Expression Array according to manufacturer's instructions (R&D systems, ARY006). For the blot, duplicate samples for each cytokine are displayed according to the coordinates provided in the Product Datasheet on the manufacturer's website (https://www.rndsystems.com/products/proteome-profiler-mouse-cytokine-array-kit-panel-a_ary006). Cytokines with no expression were omitted from the heatmap.



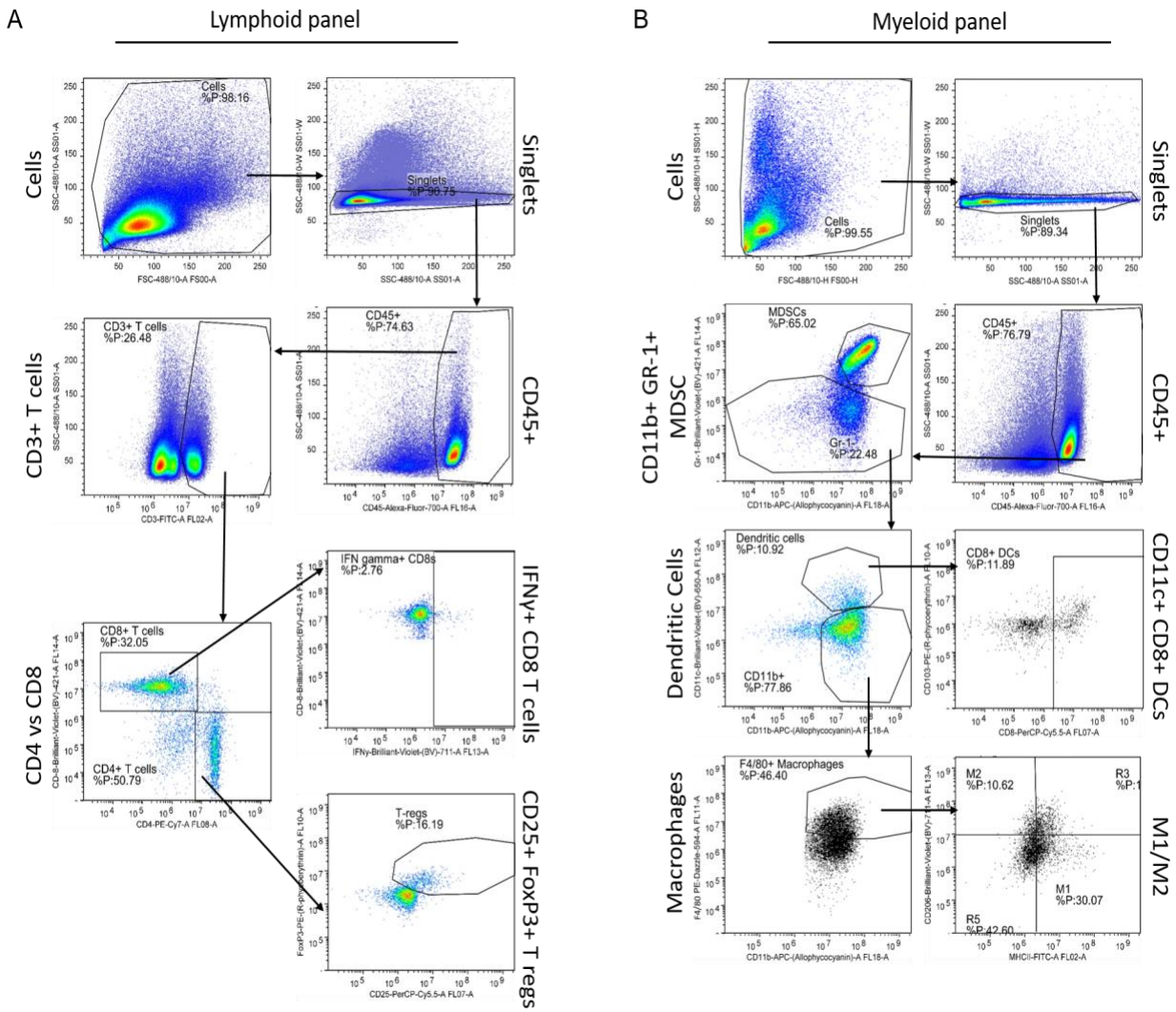
Supplementary Figure 3. Analysis of systemic cytokines. C57BL/6 mice with SQ KPC tumors were subjected to IRE (n=5) or assigned to the control group (n=5), and blood was collected from the submandibular vein on days 1, 7 and 14 after IRE. The IRE-treated mice were classified as responders (n=2) and non-responders (n=3). Plasma was isolated from the blood and the cytokine levels in them were analyzed using multiplex bead capture based assay system (Procartaplex, Luminex) and quantified using a Magpix 200 instrument. Absolute quantification was performed based on a predetermined assay standards according to manufacturer's instruction. **A** and **B** show the plasma levels of CXCL1 and IFNγ respectively in control vs IRE group. Within the IRE group, the responders and the non-responders had different plasma expression levels of cytokines CXCL1 and IFNγ (**C** and **D**). Values are represented as mean ± SEM, statistical analysis was performed using 2-tailed Student's *t*-test, ns - not significant.



Supplementary Figure 4. Circos plot showing the observed mutations in the KPC4580P tumor. First level (right next to cytogenic bands): all of the somatic mutations identified by whole exome sequencing. Second level: mutations expressed by RNAseq. Third level: histogram showing the level of RNA expression. Fourth level: 44 mutations selected for peptide synthesis and ELISPOT based on high RNA expression and sequencing depth. Innermost: gene names pertaining to peptides with positive ELISPOT response.



Supplementary Figure 5. Tumor growth in response to IRE when both the primary and the secondary tumors are implanted simultaneously SQ on contralateral flanks of C57BL/6 mice. There was no response to IRE in either the treated tumor **(A)** or the untreated secondary tumor **(B)**. **(C)** Tumor growth response to 1V270 and 1V270+ anti-PD-1 combination (using same dosing regimen as in Figures 5 and 6) compared against IRE alone and control group (n=5).



Supplementary Figure 6. Representative dot plots from flow cytometry analyses performed in Figures 2, 5 and 7 showing the gating strategies used to identify the different immune cell subpopulations in the lymphoid compartment **(A)** or the myeloid compartment **(B).**

Supplementary Table 1. List of flow cytometry antibodies (all rat anti-mouse unless otherwise mentioned)

Antigen	Fluorophore	Clone	Source
CD45	AF700	30-F11	BioLegend
CD3	BV605/FITC	17 A2/145-2C11	BD
CD4	PE Cy7/BB700	GK1.5	BD
CD8	BV421/PE-Cy7	53-6.7	BD
CD11b	APC/FITC	M!/70	BioLegend
CD11c	APC-Cy7/PE-Cy7	HL3/N418	BD
Gr-1	BV421	RB6-8C5	BioLegend
MHC II	FITC	M5-114.15.2	Tonbo
CD206	BV711	C068C2	BioLegend
F4/80	PECF594/PE	T45-2342/BM8	BD
FOXP3	PE	MF23	BD
CD25	PerCP CYy5.5	PC61	BioLegend
IFNγ	BV711	XMG1.2	BD

Supplementary Table 2. List of IHC antibodies

Antigen	Conjugate	Clone	Source
CD45	-	Polyclonal	Abcam
CD4	-	EPR19514	Abcam
CD8	-	4SM16	Thermofisher